

# ENERGETICS OF THE HOST-DEPENDENT *MYCOBACTERIUM LEPRÆMURIUM* DURING TRANSITION TO A CAPACITY FOR EXTRACELLULAR GROWTH

A. M. Dhople and J. H. Hanks

*Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory  
Baltimore, Maryland*

The possibility of cultivating so-called host-dependent microbes (HDM) is fascinating. We now focus on *Mycobacterium lepraemurium*, the previously "obligate intracellular agent" of rat leprosy. Since this organism, until recently, had been noncultivable for 70 years, it is a model from which one can derive principles applicable to the urgently needed cultivation of *M. lepraemurium*. Metabolic or biochemical monitors of the physiologic state of the experimental materia are a sine qua non because (1) such methods measure inhibitory conditions as readily as favorable ones, and (2) the lack of normal enzyme content makes host-dependent organisms particularly susceptible to inhibition.

In order to develop a biologically significant biochemical tool for quantitating the energetics and growth potential of unwashed, host-grown microbes during the progression, regression, and therapy of diseases such as leprosy, we have taken advantage of the fact that ATP can be measured in picogram amounts; that it is the source of energy for biosynthesis; and that ATP data can be interpreted either in terms of functional biomass or growth potential as follows:

- ATP pools are extremely labile, either used rapidly for biosynthesis, exchanged with related nucleotides, or in damaged cells, degraded rapidly by ATPase.
- Under constant conditions (for example, in vivo), the ATP pools within bacterial cells are controlled by the net balance between rates of generating energy and rates of biosynthesis. ATP per aliquot or per culture measures functional biomass or cell numbers.
- Minimal levels of ATP suffice for energy of maintenance; maximal levels promote maximum growth rates. Thus, ATP per bacterial cell can rank suspensions of a given species in terms of growth potential.

Because ATP data afford a means for distinguishing between genuine and spurious microscopic growth, the ATP system was utilized to investigate two reports of the microscopic growth of *Mycobacterium lepraemurium* in extracellular environments. In order to standardize conditions for incubations both in vitro and in mice, all inoculums consisted of a few million *M. lepraemurium* cells enclosed in Rightsel-Ito type diffusion chambers.

Oiwa in 1967 reported slow growth of *M. lepraemurium* when adsorbed onto siliconed slides and supplied daily with a medium containing 10 percent serum and filtered mouse brain extract. All components were prepared and handled as for mammalian cell cultures. In procedure 1 it is seen that this medium provided redundant sources of nitrogen and vitamins and practically everything required by fastidious microbes or mammalian cell cultures.

In the Rightsel system, the chambers were implanted in the mouse peritoneal cavity for 50 days before being analyzed. The results obtained in these two types of experiments are presented in figure 1.

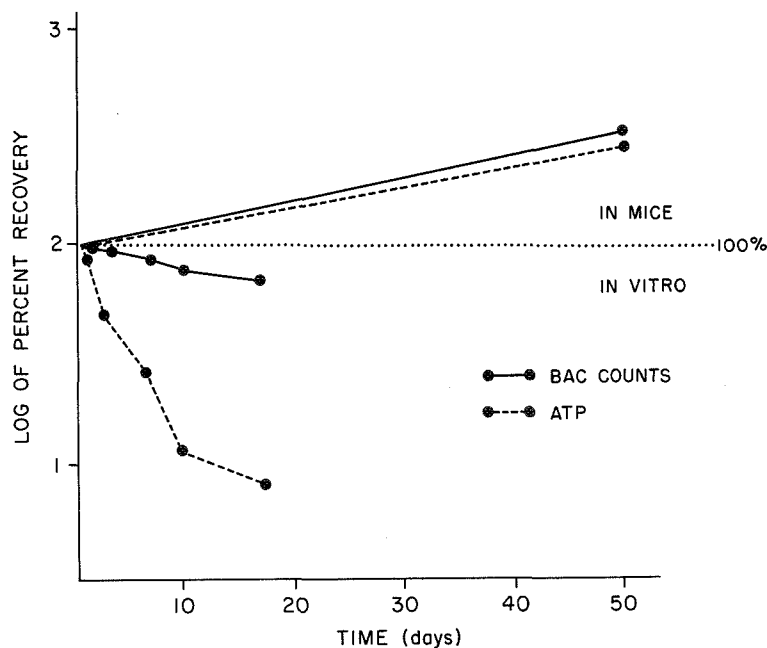


Figure 1. Bacteria counts and ATP in extracellular *M. lepraemurium* cells incubated in mice and in vitro.

In the Oiwa medium (see figure 1), irrespective of incubation system, modifications of brain extracts, or frequency of renewals, the energetics of the

Procedure 1  
Preparation of Basal Medium and Brain Extract

Ingredients	Original (Oiwa)	Modified (Basal Medium)
$\text{KH}_2\text{PO}_4$	4.0 g	---
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.0	---
Sodium glutamate	1.0	---
Succinic acid	0.2	---
Magnesium sulphate	0.6	0.6 g
Fe Ammonium citrate	0.4	0.4
Sodium citrate	2.0	2.0
L-Asparagine	2.0	2.0
L-Glutamine	1.0	1.0
Casamino acids	2.0	2.0
Glucose	10.0	10.0
Sucrose	76.0	76.0
Yeast extract	5.0	5.0
Cytochrome - c	20 mg	20 mg
Ca-pantathionate	200	200
ATP	200	200
Distilled water to make	1,000 ml	900 ml
pH	6.8	6.8
Sterilization	Seitz	Seitz
Add aseptically:		
Penicillin (10,000 $\gamma$ /ml)	1.0 ml	1.0 ml
Bovine serum	100 ml	100 ml

---

Brain Extract

Remove brains from young mice; prepare 10 percent (w/v) extract in the above medium; centrifuge for 60 min in cold at 2,000  $\times$  g; supernate turbid.

Centrifuge the supernate for 2 hours in cold at 18,000  $\times$  g; supernate clear.

Portion of the above brain extract pasteurized by heating at 333 K (60°C) for 30 minutes.

Keep frozen until ready to use.

*M. lepraemurium* cells declined steadily and uniformly. After 17 days of incubation, 72 percent of the original number of bacilli was recovered. Meanwhile, the ATP per cell had decreased to 9 percent of the original values, leaving no possibility that genuine growth could occur.

Very different results were obtained from chambers incubated by Rightsel in the peritoneal cavities of mice. After 50 days, the bacterial biomass had increased 2.7-fold and the ATP per culture 2.5-fold. Since ATP per cell was 93 percent of the original, the Rightsel system is regarded as the first to permit extracellular growth of a so-called "obligate intracellular microbe." It follows that *M. lepraemurium* is capable of being cultivated in bacteriologic media.

In 1972, Nakamura (see procedure 2) from Japan reported that *M. lepraemurium* undergoes microscopic growth in a nonconventional system, characterized by restricted air volume and the inclusion of l-cysteine in the medium. The autoclaved base, to be used as a control medium, is adequate for the growth of tubercle bacilli. The filtered supplements for the complete medium, NC-5, are listed in the right-hand column of procedure 2.

Procedure 2  
The Nakamura System

A. Physicochemical:			
6 ml of medium in #13 S.C. tubes leaves 34 percent air space.			
7 mg percent of l-cysteine, HCL.			
B. Components:			
EK = Base salts +	%	NC - 5 = Base + supplements below	%
Na-glutamate	0.90	$\alpha$ -ketoglutaric acid	0.120
Na-pyruvate	0.30	Cytochrome C	0.012
Glucose	0.25	Hemin	0.003
Glycerol	2.00	l-cysteine, HCL	0.007
Ca-pantothenate	0.006	Serum	10.0/vol
pH = 7.0		pH of all = 7.0	
Autoclave 121° - 15 min.		All filtered through	
pH = 7.0		Millipore MF, 0.22 $\mu$ m	
Inoculate and immediately distribute 6 ml/tube.			
All percentages = final concentrations in the medium.			

Figure 2A demonstrates why quantitation of functional biomass (F Bm) is more instructive than microscopic counts or, indeed, plate counts of microbial species that possess a high plating efficiency. When the in vivo grown cells of *M. lepraemurium* are inoculated in the Kirchner semisynthetic base (EK), the F Bm of *M. lepraemurium* fell to 50 percent of the original at the third day

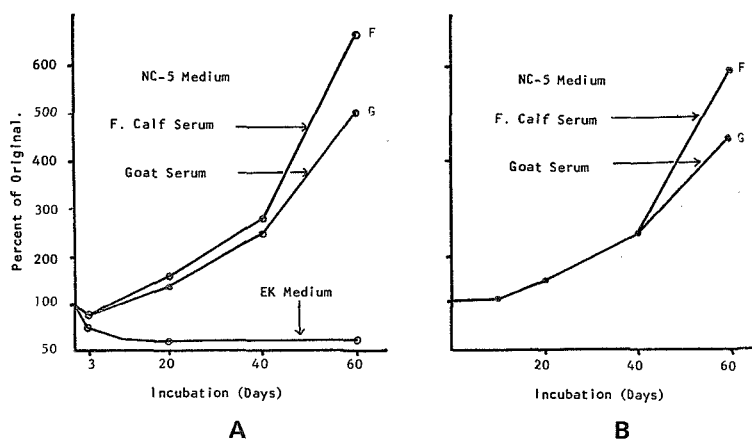


Figure 2. A—ATP per culture. B—Biomass per culture.

and declined progressively thereafter. This curve duplicates the slopes shown earlier with the mouse brain extract medium. Meanwhile, in the presence of the physicochemical conditions and catalysts provided by the supplements in the Nc-5 medium, the F Bm falls only to 75 percent of the original, that is, one half as far. Thus, within three days, it was known that the critical conditions and catalysts of the Nakamura system resided in the supplements, not in the base. By the 10th day in the complete medium (Nc-5 curve), the cultures had reestablished the energy levels utilized by *M. lepraemurium* for growth in mouse tissues, that is, the cells had successfully reconstructed the leaky in vivo type membranes into those which are competent for extra-cellular growth. On the NC curve between the 4th and the 10th day, *M. lepraemurium* already had spent 6 days in expanding its metabolic equipment and membrane activity and possibly had accomplished increases in cell volume.

Within 20 days the rate of expanding F Bm had improved slightly. After 20 days fetal calf serum is shown to be superior to goat serum. Thus, the goals of such experiments had been accomplished within three weeks.

After 10 days of lag (figure 2B), microscopic measurements of total biomass (cell numbers  $\times$  their average length) increased in parallel with the ATP curves, but the cultures had to be incubated at least twice as long to guarantee that significant growth had occurred. Experience has shown that microscopic quantitations of the total Bm of *M. lepraemurium* cultures is exceptionally tedious. *M. lepraemurium* is one of the mycobacterial species that elongate during the early stages of growth. If the work required to enumerate bacterial cells by the pin head method is regarded as one unit, determination of the average length of the cells requires three units of effort. In short, valid microscopic measurements of the biomass of *M. lepraemurium* per culture requires not one, but four units of labor.

Physiochemically, *M. lepraemurium* hitherto has proven to be noncultivable in many thousands of experiments of dozens of investigators. Any system which permits the growth of such an organism in vitro will be nonconventional in some definable respect. In the Nakamura system (see table 1):

- The unique feature is in filling 66 percent of the tube space with a medium containing cysteine.
- In the lower right section of table 1 is shown that, when the ratio of air in the tube was increased, the onset of growth was severely repressed.

The left section of table 1 ranks the contribution made by each compound in the medium.

In order to facilitate investigations with host-grown microbes we outline two propositions:

1. Metabolic or biochemical indicators of physiologic states are more significant than microscopic or plate counts, because (a) such methods measure inhibitory conditions as readily as favorable ones, (b) they help to study the physiologic sag and thus the essentials can be learned without waiting for growth to occur, and (c) the lack of normal enzyme versatility makes host-dependent organisms particularly susceptible to inhibitions.
2. Given useful sources of both nitrogen and carbon and relatively constant conditions, ATP per culture measures F Bm, the parameter that should correlate with plate and microscopic counts.

Physiologic monitors are required to analyze the transition of in vivo-grown microbial cells from noncultivable to cultivable state. Even assuming the investigation of any microbe with high plating efficiency, plate or microscope counts cannot analyze the crucially important events which are fundamental to the adaptation of a host-dependent microbe to existence in vitro. Thus, the physiologic sag of the host-dependent microbe has been defined as analogous to, but far more severe than, the sag or lag which occurs when minimal inoculums of cultivable microbes are transferred to a new medium. The observations here demonstrated that measurement of F Bm produces information of the type that cannot be gained by plate or microscopic counts, also that significant results can be obtained during half the incubation time required to obtain the earliest information by means of biologic methods.

In conclusion, I wish to summarize two points.

1. It appears that ATP measurements have been refined to a point where the growth potential of microscopic samples of so-called

Table 1

The Merits of Conditions and Components  
of the Nakamura System

Item	% of NC-5	Rank	Item	% of NC-5	Rank
NC-5	100				
$\alpha$ -Ketoglutaric	62	1	Glycerol	6	7
Hemin	59		Glucose	5	
Cytochrome C	41	2	Depth of medium	% of air space	% of NC-5
FCS	40				
l-cysteine, HCl	29	3			
Na-pyruvate	24	4	6 ml	34	100
Ca-pantothenate	18	5	5 ml	45	88
Na-glutamate	15	6	3 ml	67	52
			1 ml	89	32

Method: Starting with the complete NC-5, each item was deleted in turn.

% of NC-5 = the percentage gain when each item was restored to the deficient medium.

host-dependent microbes can be measured, even before an increase in microbial numbers has occurred.

2. This tool differs from other biochemical indicators of physiologic states in its sensitivity and in the fact that it can be exploited with unwashed host-dependent and host-grown organisms. We trust that the present studies have created new means of investigating the energetic integrity and the biosynthetic potential in *M. leprae* during experiments of the type demonstrated and also during the progression, regression, and therapy of the disease in humans.